Establishment and Characterization of Innovative Immunoreagents for Diagnosis of Neurodegenerative Disease

Tove Persson
Department of Immunotechnology
LTH, Faculty of Engineering, Lund University
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Supervisors Fujirebio Diagnostics AB:
Urszula Gädke, urszula.gadke@fdab.com and Catharina Wising, catharina.wising@fdab.com

Supervisor Lund University:
Christina Sakellariou, christina.sakellariou@immun.lth.se

Examiner:
Mats Ohlin, mats.ohlin@immun.lth.se

Popular Science Summary

Imagine a world where brain diseases like Alzheimer's, ALS, and MS, collectively affecting millions worldwide, could be met with an early diagnosis and thereby timely treatment. Over 50 million individuals live with dementias, granting many more affected by the diseases. As advancements in treating these diseases emerge, the spotlight intensifies on the value of early diagnosis. Early detection could not only extend lives but contributes to a richer, longer journey shared among family and friends.

In our immune system, antibodies act as an alarm system which tracks down invading agents like viruses and thereby makes sure that we humans stay healthy. Each antibody has sites on its surface which recognize specific sites on the invading agent. Scientific progress has made sure that we can use antibodies and their specific alarm mechanism outside of the body, both in diagnostics and in treatments. The use of antibodies to detect compounds in a sample is called an immunoassay. In this thesis, antibodies are used in an immunoassay to detect a compound called Neurofilament Light (NfL).

Why NfL? It is because when suffering from brain-affecting diseases like Alzheimer’s and others, the brain cells get damaged and NfL compounds emerge from the neurons to the blood and cerebrospinal fluid (CSF). By measuring the concentration of NfL in a person’s blood or CSF, one can determine if this person is sick in a brain-affecting disease.

The study showed some positive results proving that the antibodies used could indeed detect the NfL compound in different patient samples and that further optimization of the tests are possible. Maybe this small piece of science can be a contribution to the utopic world were brain disease is detected even before its first sign and were we conquer it with early and correct treatment.

Abstract

Neurodegenerative diseases, including Amyotrophic Lateral Sclerosis (ALS), Alzheimer's Disease (AD), and Multiple Sclerosis (MS) emphasise the need of effective diagnostics tools to provide early diagnosis and disease prognosis as well as treatment. The Neurofilament Light Chain (NfL) antigen is a promising biomarker in immunoassays for neurodegenerative disease diagnosis due to its elevated concentration in Cerebrospinal Fluid (CSF) during axonal damage. In this study, novel antibody-based reagents for an automated chemiluminescence immunoassay (CLIA) were developed towards the NfL antigen in CSF using bioconjugation techniques. The diagnostic potential of the immunological test was evaluated by comparison with an established CSF NfL assay of another CLIA instrument, Lumipulse. Capturing antibodies were coated to paramagnetic particles (PMP) and detecting antibodies were conjugated to Alkaline Phosphate molecule (ALP). When combined in five different combinations, the results showed varying recovery rates from 0 – 161% compared to the predicate device. Two reagent combinations showed promising results, with mean recovery rates of 91% and 113% respectively, indicating potential future optimisation of the test. It was established that the reagents showed diagnostic potential with the right assay configuration and pairing.

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Abbreviations

ALP	Alkaline Phosphatase
AD	Alzheimer’s Disease
ALS	Amyotrophic Lateral Sclerosis
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CLIA	Chemiluminescence Immunoassay
CDR	Complementarity-Determining Region
FDAB	Fujirebio Diagnostics AB
IFU	Instruction for Use
IF	Interfilaments
mAbs	Monoclonal Antibodies
MS	Multiple Sclerosis
AHTL	N-acetyl Homocysteine Thioacetone
Nf	Neurofilament
NfL	Neurofilament Light
PMP	Paramagnetic Particles
PD	Parkinson’s Disease
PNS	Peripheral Nervous System
RLU	Relative Light Unit
UHPLC-SEC	Ultra High-Performance Liquid Chromatography based Size Exclusion Chromatography

Introduction

Neurofilament Light Chain (NfL) is one of four subunits of the protein Neurofilament (Nf), a protein in the neurons of the human Central Nervous System (CNS). NfL functions as cytoskeleton structural support and proliferates radial growth of axons, consequently maintaining electrical impulse transmissions. During neurodegenerative diseases, such as Alzheimer's Disease (AD), Amyotrophic Lateral Sclerosis (ALS), Multiple Sclerosis (MS), and Parkinson's Disease (PD), axonal damage is occurring which leads to elevated levels of NfL in body fluids like blood and cerebrospinal fluid (CSF) [1]. The high levels of NfL makes it a noteworthy biomarker for neurodegenerative diseases.

As the medical therapies for neurodegenerative disease are developing at a rapid pace, so does the need for early and accurate diagnosis. Immunoassays are a way of quantifying biomarkers with the use of antibodies and thereby being a valuable tool in diagnostics. In this study, antibodies are treated with bioconjugation techniques to be used in a chemiluminescence immunoassay (CLIA) to establish their relevance as reagents towards NfL. The capturing antibodies are coated to paramagnetic particles (PMP) and the detecting antibodies are conjugated to Alkaline Phosphatase molecules (ALP). The diagnostic potential of the reagents is further evaluated in an automated medical device by comparison with the predicate device, Lumipulse manufactured by Fujirebio.

Fujirebio Diagnostics AB

The experiments for this research project were conducted at the facilities of Fujirebio Diagnostics AB (FDAB) in Mölndal, Gothenburg. FDAB originated as CanAg, a spin-off company that emerged from a research group at Gothenburg University in the 1980s. Subsequently, CanAg was acquired by the American company Fujirebio Inc., leading to FDAB's integration into the Fujirebio Holdings group, which has global divisions, including Fujirebio in Japan, and Fujirebio Europe in Belgium. Today FDAB and the rest of the Fujirebio Group is a R&D-driven biotech company which develops and provides in vitro diagnostic products to laboratories and hospitals around the world. Initially, their primary focus has been the production and development of cancer tumour biomarkers, but the company is now advancing in producing biomarkers for neurodegenerative diseases as well.

Previous work

In advance of this project, antibodies with diagnostic potential have been selected as a result of prior in-house knowledge. The antibodies have been manufactured by immunisation of mice with bovine NfL.

Limitations

Experiments and analysis have been carried out at FDAB with in-house protocols and instruments and materials available on site. Limited amount of patient material was available which caused some controls to not be able to be analysed in the predicate device. Limited variation of patient samples was available which, along with the limited sample amount prevented full evaluation of the diagnostic potential of the test. Additionally, time constraints contributed to limited possibility of confirming or optimising the methods and reagents.

Aim of the study

This study aims to develop a CLIA towards the biomarker NfL in CSF with antibody-based reagents using bioconjugation techniques. The reagents will be integrated into a CLIA, and their functionality will be assessed using an automated medical instrument. Additionally, the diagnostic potential of the developed immunoassay will be evaluated by result comparison with a predicate device, i.e., another medical instrument, which possesses an established CSF NfL assay on the market.

Background

Neurofilament Light

Intermediate Filaments (IF) are intracellular polymers that possess two important functions; structural support and regulation of cellular processes such as growth, proliferation, and apoptosis, through interactions with various cellular proteins [2]. IFs can be divided into six classes, where Neurofilament (NF) belongs to class IV. The subunits of Neurofilament are called Neurofilament Light (NfL), Neurofilament Medium (NfM), and Neurofilament Heavy (NfH) from their structure and molecular mass. α-internexin is the fourth NF subunit in the central nervous system (CNS), and peripherin is the fourth NF subunit in the peripheral nervous system (PNS) (**Figure 1**). Neurofilaments are essential for the radial expansion and stability of axons. Additionally, they play a critical role in enhancing the conduction velocity of electrical impulses by increasing axon diameter and modulating ion channels [3].

The domain structure of the NF subunits is similar. NfL consists of a head domain rich in serine and threonine residues and contains glycosylation and phosphorylation sites for post-translational modifications. The central rod domain is made up of a conserved alpha-helix. The tail is the domain that mainly distinguishes the four subunits due to its length of glutamic-rich and lysine-rich segments. The NfL unit has a short tail. NfL composes the backbone of the NF and together with the three other subunits, they form a complete NF with the aid of ionic strength, pH, and temperature (i.e., not by nucleotide linkage or hydrolysis). The assembly of NF subunits, including NfL, NfM, NfH, and α-internexin (or peripherin), undergoes dynamic changes throughout axon development [3].

During disease or damage, NF proteins are released from axons. Therefore, their elevated levels in blood or CSF can be used as biomarkers of axonal injury, axonal loss and neuronal death, all of which are signs of neurodegenerative diseases [3]. NF subunits are, in addition to being diagnostic markers, potential prognostic markers for ALS, MS, and for assessing nerve damage in patients with severe burns. In a recent study, elevated NfL levels in CSF showed a correlation with frontotemporal dementia severity [3].

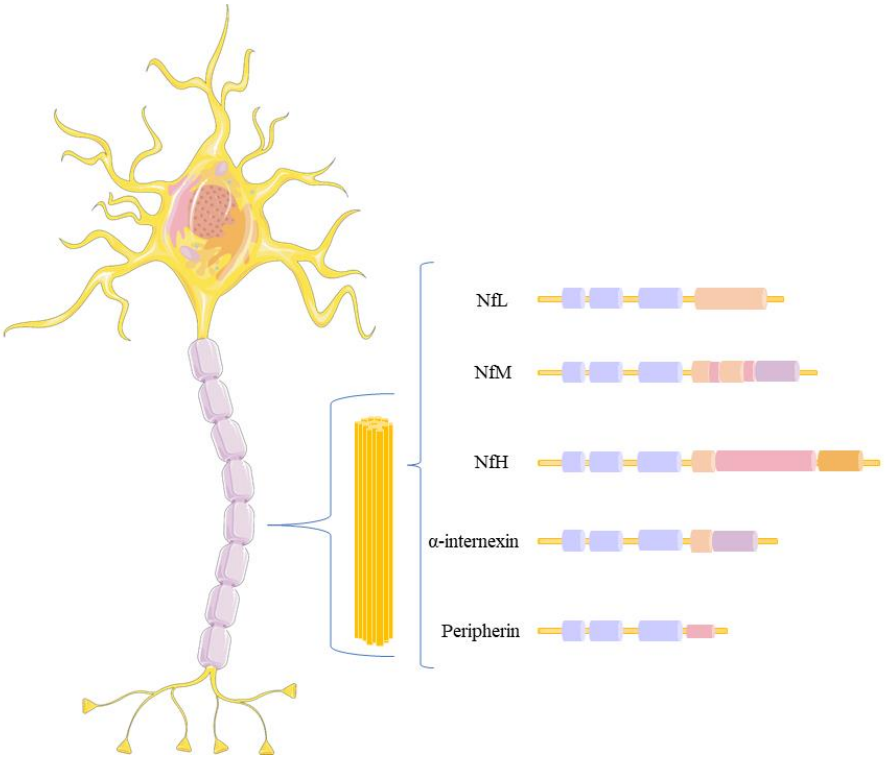


Figure 1. Schematic view of a neuron and neurofilament structure and subunits. Figure constructed in PowerPoint with image from SMART.

The knowledge of the molecular composition and post-translational modifications of NfL in bodily fluids is currently limited. However, existing reports suggest the presence of various NfL species in CSF samples. Research indicates that in the CSF of patients with neurodegenerative diseases, various fragments of the NfL compound are present, while full-size NfL proteins are predominantly found in brain tissue [4].

A recent study also revealed the tendency of NfL to dimerize, observed in both human CSF samples and bovine NfL-based calibrators [5]. Through SEC, this study demonstrated that the NfL in bovine NfL-based calibrators consisted of full-length NfL dimers, whereas the antigen in CSF samples were dimers of NfL fractions. These findings underscore the significance of considering the NfL origin when interpreting the results of immunoassays in this study.

Antibodies

Antibodies are crucial proteins in our immune system that recognise and battle intruding pathogens such as viruses and bacteria. Antibodies are produced and secreted when an antigen is introduced to a B cell. In addition to fighting pathogens in our immune system, antibodies can be used in diagnostics and therapeutics [6].

Antibodies, called immunoglobulins, encompass various subclasses in higher mammals, including IgG, IgM, IgA, IgD, and IgE. Distinctions among these classes arise from variations

in their amino acid sequence, size, charge, and carbohydrate composition. IgG is the most abundant immunoglobulin in mammal blood and can be further subcategorized into four classes: IgG1, IgG2, IgG3, and IgG4, based on differences in their constant regions and functional properties.

Antibody Structure

Immunoglobulins are glycoproteins with a Y-shaped structure consisting of two identical "heavy" polypeptide chains linked together with two shorter "light" polypeptide chains attached. The number of Y units can differ between the different immunoglobulins; IgG consists of one Y unit whereas IgM is a pentamer of Y units. The Y-shaped unit can be divided into subdomains: the constant domain (e.g., C_H or C_L) and the variable domain (e.g., V_H or V_L). The number of domains differs between the different Ig variants [7]. Placed on each variable region are three loop structured complementarity-determining regions (CDRs). The CDR loops are, as the name suggests, the parts of the IgG molecule which bind to the specific antigen and are thereby crucial for the antibody specificity. Small deviations in the CDR amino acid chain can result in reduced recognition and specificity [7], [8].

Antibody Origin

Antibodies may be polyclonal, monoclonal, or recombinant and the choice of which sort to use is determined by the application. Polyclonal antibodies are a mixture of immunoglobulins displaying specificity for a specific antigen, each antibody specific to a different antigen epitope [9]. Monoclonal antibodies (mAbs) are identical antibodies with binding affinity to one single epitope on the antigen. These are produced by antibody-secreting hybridoma cells. Recombinant antibodies are monoclonal antibodies, i.e. mAbs with specificity to one epitope, that can be produced via phage display and thereby avoid the continuous immunisation of animals [7].

Antibody Stability

Antibodies are one of the most stable proteins and are resistant to environmental changes due to their structure [8]. However, due to their various chemical interactions, such as van der Waals forces, hydrophobic interactions, disulphide linkages, and hydrogen bonds, antibodies tend to aggregate. Aggregation affects antibody stability during production processes, delivering and storage, hence it is important to make sure that the antibodies used in an immunoassay attain low aggregation rates [10]. Protein stability can be at risk by other things, including foreign substances in its presence and product impurity during handling and storage. In immunoassays, this can cause the loss of antigen recognition by the antibody, continuously affecting the assay performance [8].

Other parameters might affect antibody stability, namely that glycosylated antibodies are more stable than non-glycosylated antibodies [11]. Complete mAbs are more stable than free antibody fragments, i.e., fragmented Ab (Fab). The choice of buffers can affect the stability of

monoclonal antibodies and the antibody concentration is of interest. High mAb concentration is reported to reduce aggregation [10]. To optimise long shelf-life and antibody stability, careful selection of buffers, additives and excipients are required. Commonly employed protein stabilisers include BSA, suitable pH, protease inhibition and antibacterial agents [12], [13].

Immunoassay

Immunoassay is a technique for measuring the presence of micro molecules in a biological sample. In immunoassays, the unique characteristics of antibodies are used, including their ability to bind to a wide range of chemicals, cells and viruses, their specificity for the target substance, and the binding strength between the antibody and its target, here called antigen [14]. Immunoassays are highly dependent on the specificity of antibodies [15].

Immunometric Immunoassay

The immunometric immunoassay design, also called sandwich assay, is one type of immunoassay in which an antibody is immobilised onto a solid phase and thus can capture the antigen from an added sample. The antigen can then be recognised by another antibody which is labelled and therefore able to produce a signal response to enable quantification of the antigen [14].

Chemiluminescence Immunoassay (CLIA)

The principle of Chemiluminescence Immunoassay (CLIA) is explained as quantification of proteins in biological samples by measurement of chemiluminescence. Luminescence is radiation emission generated by electrons going from excited state to ground state [16]. In an immunoassay, the luminescence label can emit light directly at contact with the antigen or in reaction with additional substances after being conjugated to the antigen. In this study, the detection Ab is conjugated to the molecule ALP which, in reaction with the assay substrate, emits the chemiluminescent light. The light is thereafter detected and measured in relative light units (RLU). Moreover, the RLU response is used to quantify the antigen concentration with the use of a standard curve.

Assay Performance

To manufacture a reliable and acceptable immunoassay, optimisation of immunoassay properties is required. Multiple assay characteristics are important such as sensitivity, specificity, robustness, precision, and accuracy. These properties are fundamental to trusting the immunoassay results and are optimised during immunoassay development.

Immunoassays are subject to interference and the results that may bring. Interfering substances can interact with either the analyte or the reagents and may contribute to falsely negative or falsely positive results, i.e., decreased, or elevated analyte concentrations which in worst case may lead to misdiagnosis. Heterophile antibodies and human anti-animal

antibodies are two examples of antibody-interfering agents. Heterophilic antibodies are endogenous antibodies that are present in patient samples and bind weakly to a variety of antigens leading to faulty results.

In immunometric assays there is increased risk of bridging between the capture and detection Abs Steric hindrance may occur when Rheumatoid factors bind closely to the binding site of the reagent antibodies and thereby preventing antigen binding. To reduce non-specific binding, blocking agents can be added to the reagents for binding of their free sites or neutralisation of interfering agents [17].

Clinical Relevance

Values that indicate if a patient has normal or elevated concentration of NfL in their system are called cut-off values. These values separate healthy patients from not healthy. Since NfL concentrations are higher in CSF than in blood, the cut-off values for these sample types differ. The cut-off values found in the literature have influenced the choice of calibrators and controls for the immunological test. Studies have reported varying findings about NfL level differences due to gender, some reporting that the female population of the study had more elevated NfL levels than the male population [18], while others have reported the opposite [19]. However, papers agrees upon the considerable impact of age and how NfL levels positively correlates with increased age [19], [20], [21], [22], which thereby is or should be taken into account when determining cut-off values and developing an immunoassay. NfL concentration levels in healthy patients have been presented by Uman Diagnostics [23] and Karolinska Institutet [24] and are presented in **Table 1**. These levels are based on the CSF-based NfL ELISA by Uman Diagnostics which is a different assay technique, but the values could still be a relevant benchmark during early test development.

Table 1. Reference values for healthy NfL concentrations in CSF.

Age	Reference value (pg/mL)
< 30 years	< 380
30 - < 40 years	< 560
40 - < 60 years	< 890
> 60 years	< 1850

Predicate Device

In this study, the immunoreagents are combined into an immunoassay in an automated medical device available on site. While other medical devices obtain already CSF-based NfL assays established on the market, this device does not. To enable evaluation of the immunological test developed, an automated device from another manufacturer with CLIA technique is used for

comparison; Lumipulse. In the Lumipulse device, calibrators and controls are analysed whose values are later on used as comparison for the assay runs in the automated device.

Material and Method

The experiments were performed in the order they are mentioned in this section.

Prior to the final immunoassay runs, pre-treatment of antibodies, buffer production and a calibration curve were executed. Literature studies of the NfL antigen properties, and the cut-off range for other existing NfL immunoassays were made in order to plan the experiments. To allow comparison and validation of the results of the immunoassay runs, the calibration curve and controls were run on the predicate device Lumipulse. Lumipulse is CLIA instrument and was available at FDAB premises. Excel and an Excel-based tool were used to analyse and present data points from the calibration curve and the immunoassay runs.

Antibody Selection

Antibodies were selected based on their availability and FDAB knowledge of their potential as diagnostic reagents. Four antibodies were used and paired to compose five antibody pairs (**Table 2**). The five reagent pairs were then evaluated based on their capturing and detecting properties in the automated CLIA instrument. The five combinations are referred to as runs 1, 2, 3, 4 and 5. The antibodies evaluated are referred to as NFL01, NFL02, NFL03, and NFL04. NFL01 and NFL03 are antibodies of the sub-type IgG1. NFL02 and NFL04 are IgG2b and IgG2a, respectively. Depending on the immunisation process, antibodies may bind to different epitopes of the NfL antigen. The antibodies produced at FDAB were all proven to bind to the Coil 2-region of the NfL antigen.

Table 2. Table of the antibody combinations run in the CLIA.

Capture Antibody	Detection Antibody	Combination
NFL02	NFL01	Run 1
NFL01	NFL02	Run 2
NFL03	NFL02	Run 3
NFL04	NFL01	Run 4
NFL02	NFL02	Run 5

Limited antibody and ALP availability prevented the testing of all possible antibody combinations, and the Ab pairs were thereby determined according to prior FDAB knowledge on how the Abs bind to the NfL antigen. Ab pairing choices were additionally influenced by prior knowledge of the most promising function of each Ab, as capturing or detecting Ab, from previous in-house experiments.

Buffers

To provide a stable and favourable environment for immunoassay reagents, optimised buffers are of great importance. Buffers with the right chemical properties optimise the reaction conditions for antigen-antibody binding, enzyme activity and minimise interference and thereby contributing to a reliable immunoassay [25].

Buffers were produced for the different parts of the project, including the making of the calibration curve, PMP coating, ALP conjugation, and CLIA. The buffers were made by imitating in-house protocols for previous projects with similar needs of buffer pH, blocking, coating, washing and chemical content.

Calibration Curve

When planning the concentration levels of the calibrators to be used for the calibration curve literature research was carried out, including scientific study reports and Instructions for Use protocols (IFUs) from already existing CSF NfL assay manufacturers. It was found that the assay range varied among assays as well as the cut-off values. Concentrations of calibrator levels for CSF tests were found between 50 pg/mL and 5 000 pg/mL [26], 85.5 and 25 700 pg/mL [27]. Additionally, the Lumipulse CSF NfL assay presented an assay range of 0 pg/mL to 50 000 pg/mL which determined this study's assay range for comparison reasons. The chosen calibration points are presented in Table 3. An original stock of bovine NfL with a known concentration of 1 mg/mL was used to prepare the calibrators by dilution with prepared calibrator matrix.

Table 3. Target concentration for calibrators S0-S8 in pg/mL.

Calibrator Level	S0	S1	S2	S3	S4	S5	S6	S7	S8
Concentration (pg/mL)	0	50	250	500	2500	5000	10 000	30 000	50 000

Controls

Control levels were prepared as well, but of varying antigen origin and matrix. CTRL 1, 2, 8 and 9 were prepared at FDAB with bovine NfL in calibration matrix, CTRL 3, 7 and 10 were purchased from the same manufacturer as the predicate device, Fujirebio. CTRL 4, 5, 6, 11, 12 and 13 were patient samples. Calibrators S0-S8 and controls CTRL 1-11 were primarily analysed in the predicate device and later analysed in the device used in this study. Controls CTRL 12-13 were not analysed in Lumipulse due to limited amounts of these patient samples. The two controls had previously been analysed on another instrument, Uman Diagnostic's ELISA CSF NfL Assay [26], and these values for CTRL 12-13 are used for comparison instead of Lumipulse values (**Table 4**). The concentrations in Table 3 are target concentrations from calculations (CTRL 1, 2, 8 and 9) and predicate device values (CTRL 3-7, and CTRL 10-11) and Uman Diagnostics ELISA values (CTRL 12-13).

Table 4. Determined concentrations for CTRL 1-13

Control Level	Concentration pg/mL	Antigen	Buffer/Matrix
CTRL3	787	Lumipulse Antigen	Lumipulse matrix
CTRL7	4 056	Lumipulse Antigen	Lumipulse matrix
CTRL10	21 010	Lumipulse Antigen	Lumipulse matrix
CTRL1	200	bovine NfL	Calibrator Matrix
CTRL2	400	bovine NfL	Calibrator Matrix
CTRL8	6 050	bovine NfL	CSF
CTRL9	20 000	bovine NfL	Calibrator Matrix
CTRL11	2 100	N/A	CSF bulk
CTRL4	1 287	Patient pool	CSF
CTRL5	1 710	Patient pool	CSF
CTRL6	2 070	Patient pool	CSF
CTRL12*	4718*	Patient sample	CSF
CTRL13*	> 5000*	Patient sample	CSF

* Mean dose value acquired from Uman Diagnostics ELISA CSF NfL Assay

The patient pool samples (CTRL 4, 5, and 6) are pools of multiple patient samples from Alzheimer patients showing high levels of other Alzheimer biomarkers. The CSF Bulk is also a collection of mixed samples but from donors which are considered healthy. The high NfL concentration in this control can be explained by the samples being from older patients and eventual underlying diseases.

PMP Coating

PMP coating refers to the technique of conjugating antibodies onto a paramagnetic particle, forming a coat on the particle. All four antibodies underwent PMP coating.

The antibody is linked to the particle with the aid of carbodiimides. Carbodiimides allow the formation of amide linkages between carboxylates and amines as well as phosphoramidate linkages between phosphates and amines. Since the particle is carboxylated, the bond formed is an amide bond to the amines of the antibody. In this study, the combination of the carbodiimide EDAC (1-ethyl-3-(3-dimethylaminopropyl)) and the compound sulfo-NHS (N-hydroxysulfosuccinimide) facilitates the linkage between the carboxylated particle and the antibody. Agents that allow two molecules to bond without any additional atoms are called zero-length crosslinkers. The absence of interfering linkage molecules is advantageous since it may prevent cross-reactivity between the antibody and a crosslinking agent [28].

Firstly, the particles were washed into a new buffer, meaning that the original storage buffer was changed to another buffer. This was done with the help of the magnetic properties of the particles. A tube of particle solution was placed near a magnet to which the particles were attracted. When the particles were attached to the tube wall and separated from its storage buffer, the buffer was aspirated leaving solely the particles in the tube. The tube of particles was removed from the magnet and the new buffer was added. This cycle was repeated a few times after which the particles are considered washed and in the correct buffer for further reactions.

The EDAC-sulfo-NHS mix was added to the particles to create an amine-reactive particle surface with active ester groups. Furthermore, the antibody was added which allowed its amine groups to form amide bonds with the particle's ester groups. The pair was incubated at end-over-end rotation.

The next steps included blocking of free amino sites, removing unbound antibodies, and blocking the surface of the particles to prevent undesired binding that may contribute to assay interference. Lastly, the antibody-coated particles were washed into a storage buffer and stored until being used in the immunological tests.

ALP Conjugation

While antibodies are very specific to their target molecule, they have no properties that allow them to be directly measured in low concentrations. This highlights the need to incorporate a signal-generating technique into the immunoassay, which typically is achieved by conjugating an antibody to a molecule able to facilitate a generation of signal that can be further measured. This is the method used in this degree's project and other methods will therefore not be further explained [15].

In the herein studies immunoassays, the detection antibody needs to be coupled to a molecule that, in the presence of a suitable substrate, emits a chemiluminescent signal to enable quantification of the biomarker. ALP conjugation refers to the technique of conjugating an ALP molecule to an antibody. This is done by introducing the antibody to free thiol groups and the ALP molecule to free maleimide groups.

The properties of the conjugates are crucial to the performance of the assay, and therefore the optimization of the conjugate is a key factor in achieving the desired goals of the immunoassay, thus thiolation and maleimidisation being important segments in the conjugation experiments [15].

Thiolation

Before binding to the ALP molecule, the antibody needs to undergo thiolation, which is the process where the antibody undergoes a reaction with N-acetyl Homocysteine Thioacetone

(AHTL) and forms a thiolated antibody with free sulfhydryl groups. This is necessary to make the coupling with the maleimidised ALP molecule possible at a later stage.

The amino acid Cysteine has a thiol group on its side chain that can react with other molecules. However, in native proteins, this thiol group often undergoes intramolecular reactions and therefore becomes less available for chemical reactions with its surroundings. By introducing free thiol groups to the antibody during the thiolation step, these can react with compounds such as maleimide, disulfide, and haloacetyl groups [15].

To introduce thiols to a protein, the protein's amine-reactive group needs to be linked to a thiol group with the help of a coupling agent. Free thiols tend to dimerise due to oxidation, hence the coupling agent. The coupling agents can, for example, be S-Acetylmercaptosuccinic anhydride (SAMSA), 2-Iminothiolane hydrochloride (2-IT) and N-Succinimidyl-S-acetyl thioacetate (SATA), but in the experiments of this project, AHTL was used to attach thiol groups on the protein [15].

To determine the degree of thiolation and find the optimal range for further use in the ALP conjugation step, Ellman's Reagent (5,5′-dithiobis(2-nitrobenzoic acid)) (DTNB) was used. The Ellman assay refers to the quantification of thiol groups of a protein by the reaction between the protein's thiols and DTNB where the thiol cleaves the disulfide bond of DTNB. The reaction products are one mixed disulfide and one TNB anion (2-nitro-5-thiobenzoic acid) of which the latter has a yellow colour (**Figure 2**).

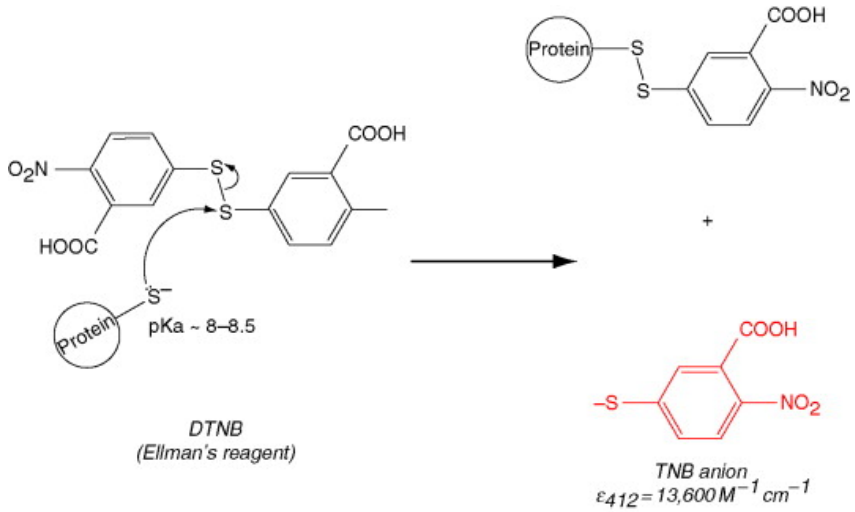


Figure 2. Ellman's Reagent reaction with a thiolated protein

The absorbance of the TNB anion is then measured at 412 nm. Since one molecule of thiol that reacts produces one molecule of TNB, the amount of signal is proportional to the number of thiols [29], [30].

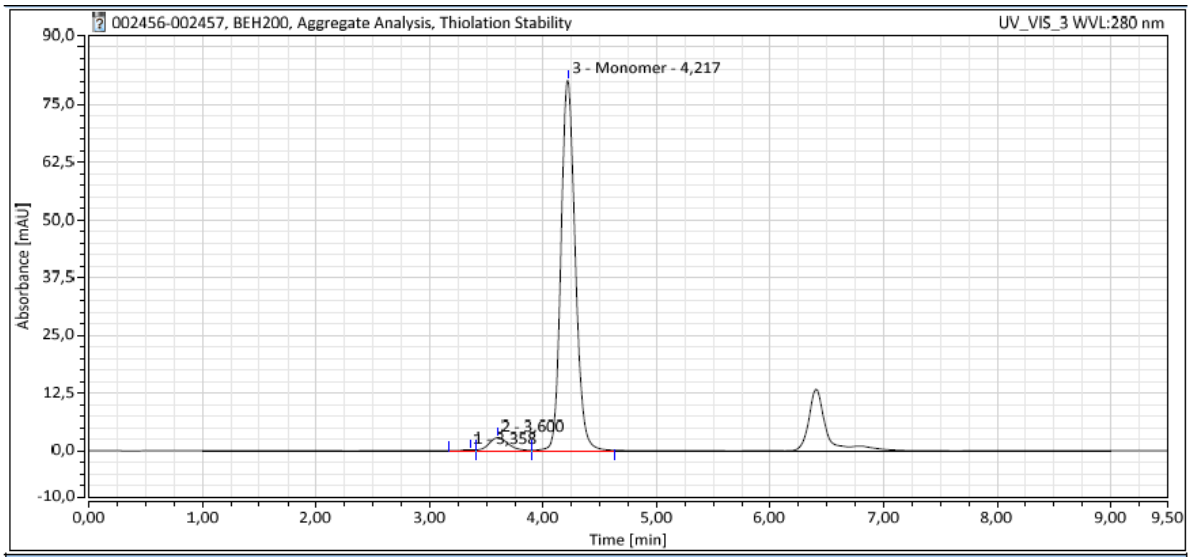
AHTL Titration

To find the optimal thiolation ratio for each thiolated antibody, AHTL titrations were performed which means that different concentrations of AHTL are used in the thiolation

process of an antibody to generate a curve. Furthermore, the curve equation can be used to calculate the concentration of AHTL needed to reach the optimal thiolation range for the antibody.

The AHTL titrations were performed by deciding three different concentration levels of AHTL that would be added to the antibodies. The procedure was performed for three antibodies; NFL01, NFL02, and NFL03.

Ensuring the stability of the thiolated antibody complex is crucial to minimise aggregation before coupling it with ALP. To assess thiol-antibody stability, Ultra High-Performance Liquid Chromatography based Size Exclusion Chromatography (UHPLC-SEC) was used to analyse the monomer ratio of thiolated antibodies. The monomer ratio, indicating the extent of antibody aggregation, is visualised by the relative change in area percentage for the antibody peak **(Figure 3).** Antibody samples were aliquoted and assessed under various conditions for comparison, including thiolated and not thiolated, storage in room temperature (RT), and 10°C, desalted (DS), and not desalted states for 0, 1, and 2 days **(Table 5).**



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Rel.Area %	Rel.Height %
1		3,358	0,0	0,2	0,18	0,20
2		3,600	0,6	2,9	4,59	3,43
3	Monomer	4,217	11,7	80,2	95,23	96,37
Total:			12,3	83,3	100,0	100,0

Figure 3. An example of UHPLC result of a thiolated antibody sample

Table 5. Scheme over UHPLC-SEC analysis

	0h	2-3h	1 day	2 days
mAb not DS	RT + 10 °C	RT + 10 °C	RT + 10 °C	RT + 10 °C
mAb DS	RT + 10 °C	RT + 10 °C	RT + 10 °C	RT + 10 °C
SH-mAb not DS	RT + 10 °C	RT + 10 °C	RT + 10 °C	RT + 10 °C
SH-mAb DS	RT + 10 °C	RT + 10 °C	RT + 10 °C	RT + 10 °C

Maleimidisation

Like the thiolation procedure, the ALP molecule needs to be processed to be able to bind to the antibody's thiol groups. This is done in the maleimidation process where ALP reacts with Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC), resulting in a derivatized ALP molecule with free maleimide groups, i.e a maleimidised ALP molecule. When the antibody is thiolated and the ALP maleimidised, the two can bind and form an antibody-ALP conjugate [15].

Conjugation

To finalise the antibody-ALP conjugation, the thiolated antibody was added to the maleimidised ALP and incubated in a heated water bath. To make sure that free thiol groups and maleimide groups do not interfere with the test, blockers are added. By artificially making sure that the antibody and the ALP molecule contain equal amounts of their respective reactive group, this approach is controllable and yields well-defined conjugates [15]. The two antibodies conjugated in this project were NFL01 and NFL02.

Immunoassay with the Reagents

When the reagents had been developed - the capture antibodies coated onto particles and the detection antibodies coupled to the signalling ALP molecules, their properties could be evaluated in the automated CLIA instrument. Five different reagent combinations were assembled by combining antibody-coated particles with ALP-conjugated antibodies as presented earlier.

The assay format was decided to be a two-step assay meaning that the sample was added to the capture antibody followed by washing, and then the detection antibody was added followed by a second wash. The sample RLU was measured in duplicate by the device and the RLU values could then be analysed and processed.

Data Analysis

An Excel-based tool from the instrument manufacturer was used to facilitate the analysis of the immunoassay reagent pairs. The tool processed the analytical data from the five assay runs and returned the calibration curve, its appropriate curve fit with the number of iterations, and dose values of the controls and samples run. Curve fitting is an important component in immunoassay performance since it has a direct effect on the dose results. The RLU response from the calibration levels are all fitted to the so-called master curve, in this case, the Lumipulse calibration curve enabling calculation of concentration doses. The tool runs the RLU responses of an assay run for comparison with the master curve, ultimately determining the appropriate model, a Four Parameter Logistic fit or a Smoothing Spline fit, and dose values. The recovery is used to compare the values with the predicate device to establish whether the immunoreagents have diagnostic potential.

Results

The results are mentioned in the same order as experiments were performed and results obtained.

Calibration Curve

The calibration levels S0-S8 were initially analysed in the predicate instrument, Lumipulse. The Lumipulse device displayed concentrations that were similar to the target concentrations **(Table 3)**, although being slightly lower for the higher calibrator levels, S4-S8 (**Figure 4**). Actual values displayed by Lumipulse, and the target values are found in Appendix I. The Lumipulse calibration curve is further used as a comparison when running the same calibrators in the automated instrument in runs 1-5 in which the reagent pairs will be evaluated (**Figure 5**).

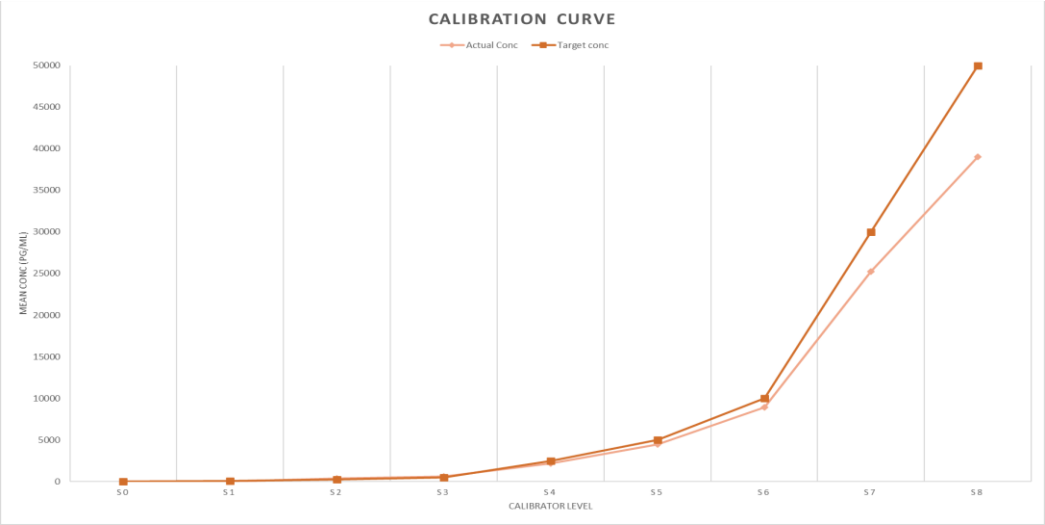


Figure 4. Graph of the calibration curve by the calibration levels S0-S8 where the darker line and the lighter line represents the target values and the Lumipulse values respectively.

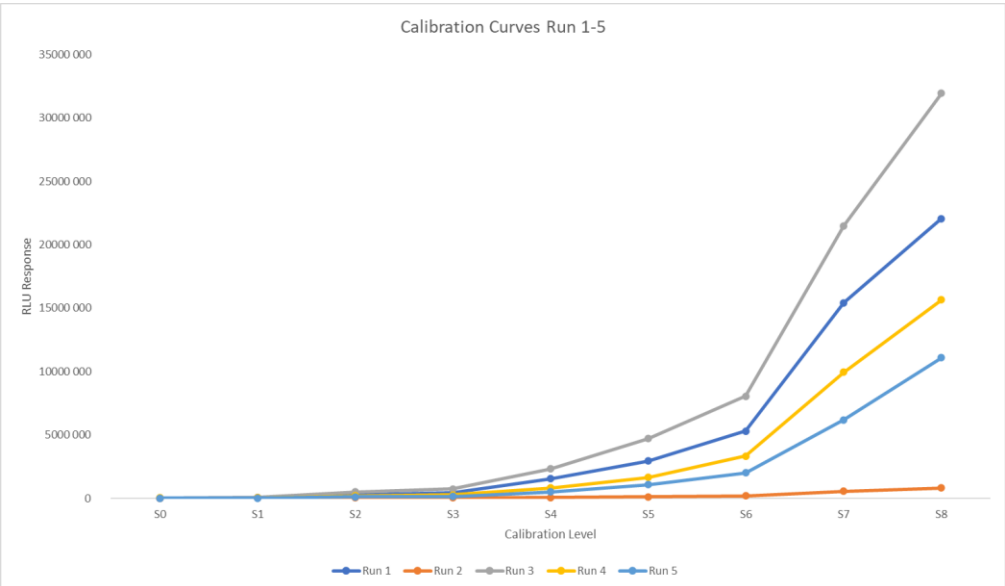


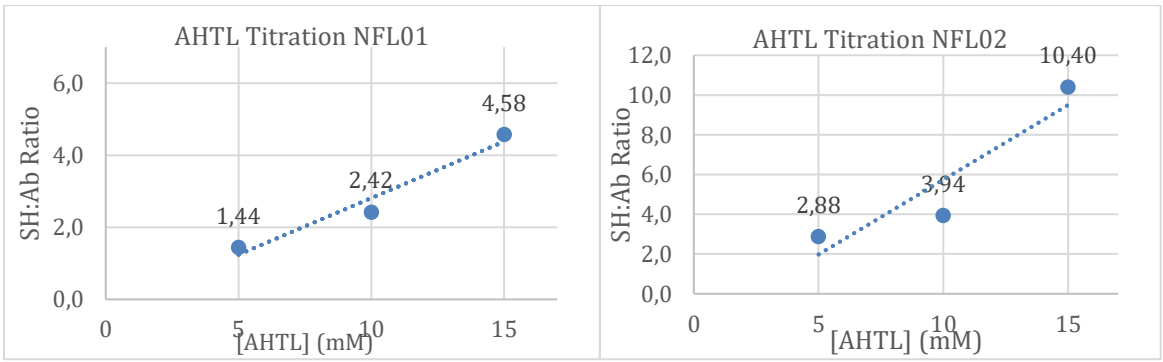
Figure 5. Calibration Curves for run 1-5 in the automated CLIA device.

ALP Conjugation

Thiolation

AHTL Titration

AHTL titration was performed for antibodies NFL01, NFL02, and NFL03. The three different AHTL concentrations to test were decided to be 5, 10 and 15 mg/mL based on prior experiments and knowledge at FDAB (**Figures 6a-c**). NFL01 showed an optimal thiolation degree at an AHTL concentration of 12.2 mM. Optimal AHTL concentration for antibodies NFL02 and NFL03 were calculated to 7 mM and 10.8 mM, respectively (**Table 5**). See Appendix II for absorbance values.



Figures 6a-b. Graphs of AHTL Titration experiments for NFL01 and NFL02.

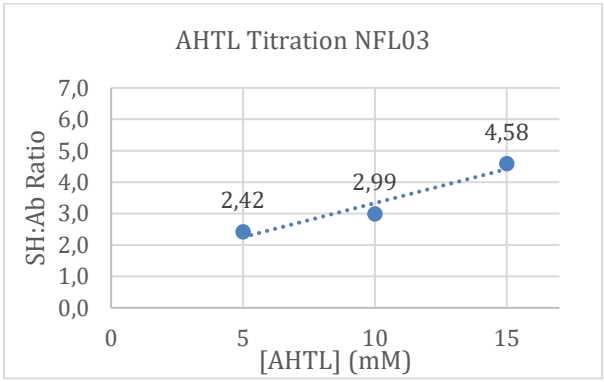


Figure 6c. Graph of AHTL Titration experiments for NFL03

Table 5. Calculated values of required AHTL concentration to achieve optimal SH:Ab ratio.

Antibody	NFL01	NFL02	NFL03
AHTL Concentration	12.2 mM	7 mM	10.8 mM

UHPLC-SEC was used to analyse the monomer ratio, and thereby the stability of the three thiolated antibodies. The results are visualised as percentage of the relative area of the peaks in graphs for each AHTL titration experiment (**Figures 7a-c).**

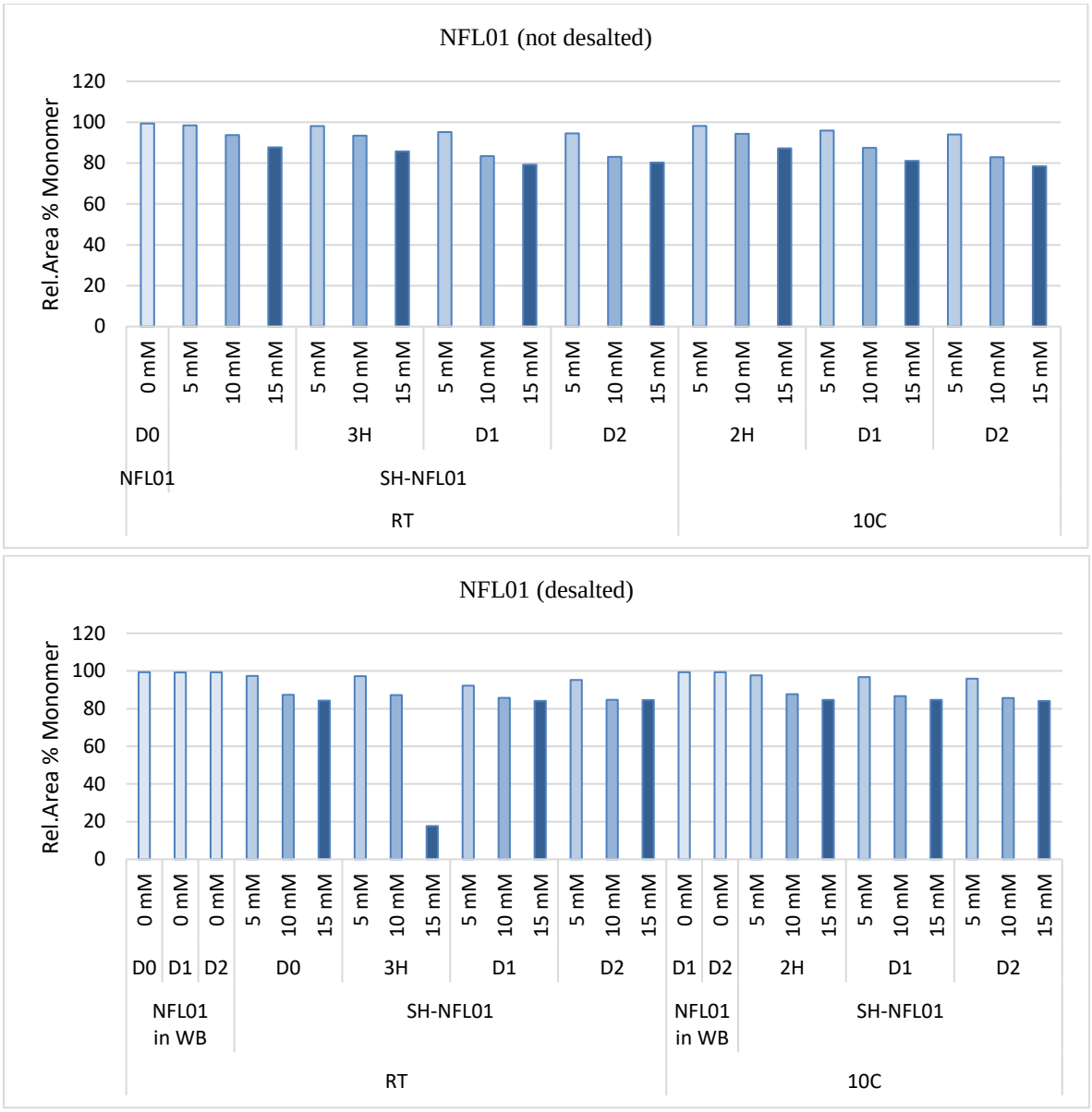


Figure 7a. Bar charts of UHPLC-SEC results from AHTL Titration experiment with NFL01

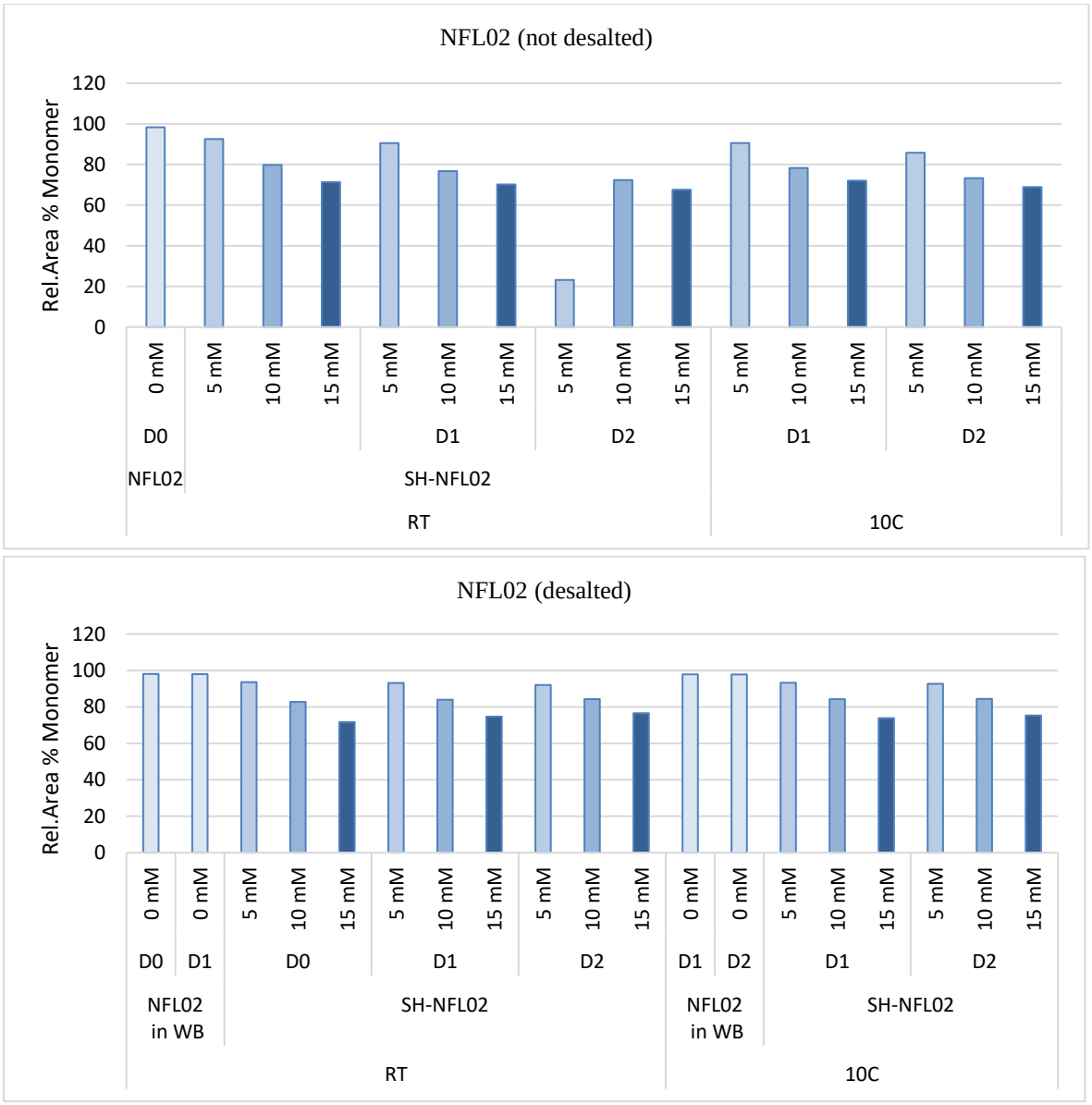


Figure 7b. Bar charts of UHPLC-SEC results from AHTL Titration experiment with NFL02

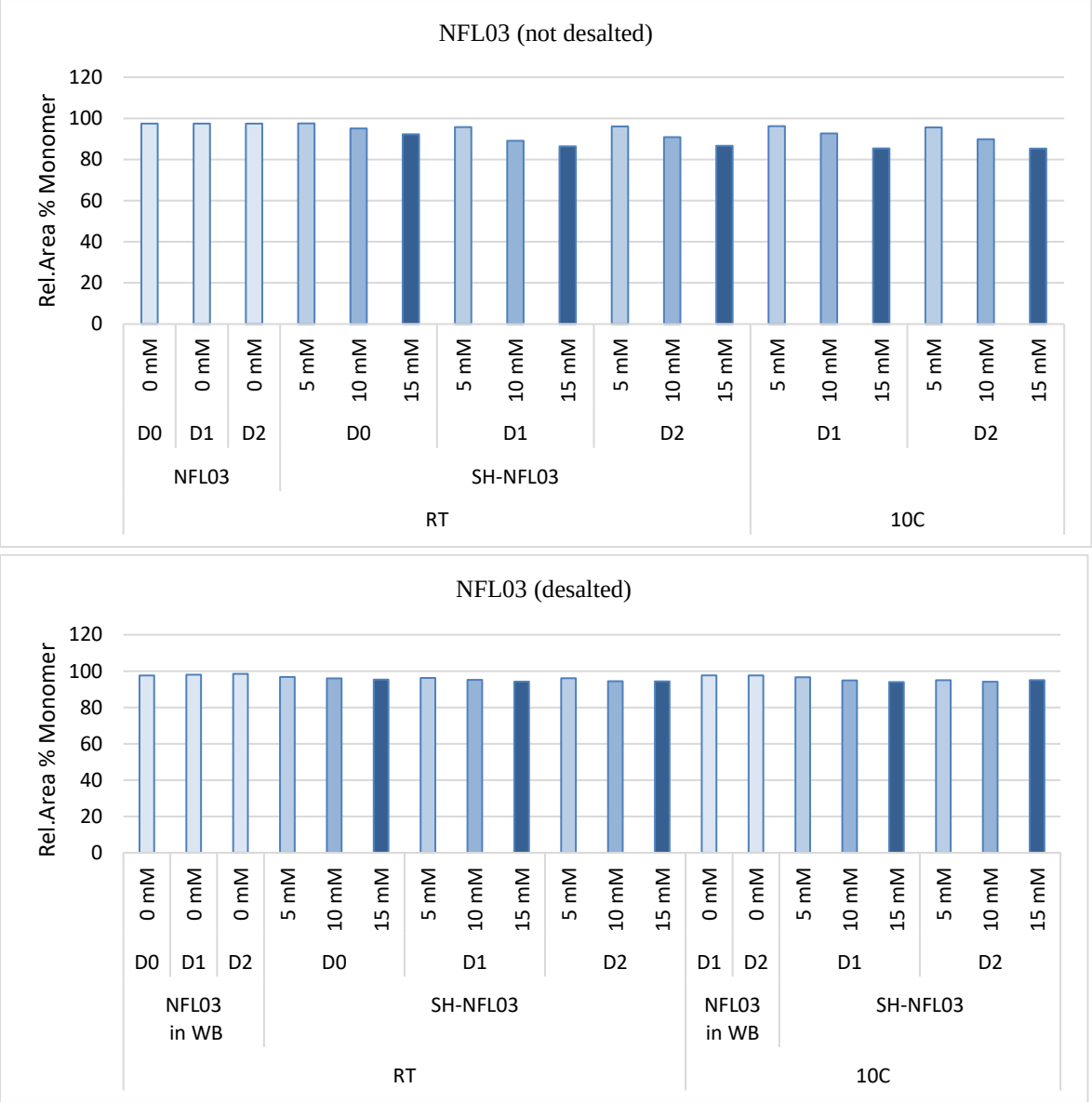


Figure 7c. Bar charts of UHPLC-SEC results from AHTL Titration experiment with NFL03

Tables of all stability values for NFL01-03 are found in Appendix III. The results show higher stability for desalted complexes than not desalted for all three antibodies. NFL03 stability is the highest, showing low change in monomer area under various conditions, while NFL02 display low stability.

ALP Conjugation of antibodies NFL01 and NFL02

Due to limited availability of ALP, only two antibodies could undergo conjugation, NFL01 and NFL02. The antibodies were thiolated with the calculated optimal AHTL concentration found in Table 5 and the ALP was maleimidised to an optimal maleimidisation degree. The two thiolated antibodies were then added into two separate flasks of maleimidised ALP and further on filtered on a SEC column to separate reactants from the wanted conjugates, C01 and C02. The result of the chromatography can be seen in **Figure 8**. The first peak, at around 70 mL of eluted solution, represents the conjugate. The second peak contains both unreacted ALP and

antibodies since they are of similar molecular weight. The third and last peak portrays eluted salts.

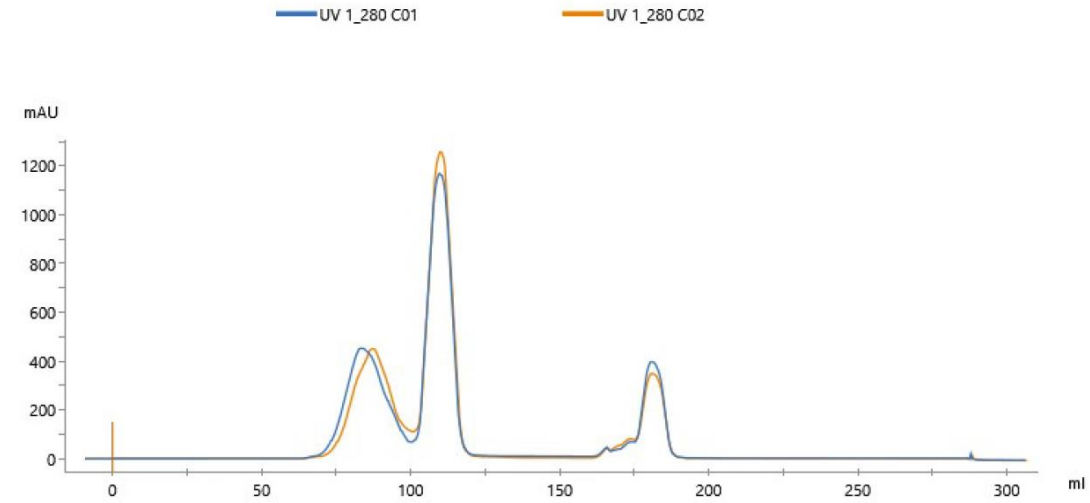


Figure 8. Size exclusion chromatography column results. Blue and yellow lines represent NFL01 conjugate (C01) and NFL02 conjugate (C02) respectively.

The eluted conjugate peak consists of a mix of conjugates of varying molecular weight. To be able to analyse what conjugate size is optimal for coming assay runs, three pools of fractions are assembled for each conjugate (**Figures 9 and 10).** The pooled fractions of the first conjugate, C01, are called C01-A, C01-B and C01-C. For the other conjugate, C02, the naming procedure is the same **(Table 6**).

Table 6. Molecular weights in kDa of conjugate pools

Conjugate pool	Molecular weight	Conjugate pool	Molecular weight
C01-A	700 - 259 kDa	C02-A	713 - 288 kDa
C01-B	544 - 259 kDa	C02-B	555 - 288 kDa
C01-C	426 - 259 kDa	C02-C	447 - 288 kDa

Chosen fraction ranges can be seen in Figures 10 and 11 for C01 and C02, respectively.

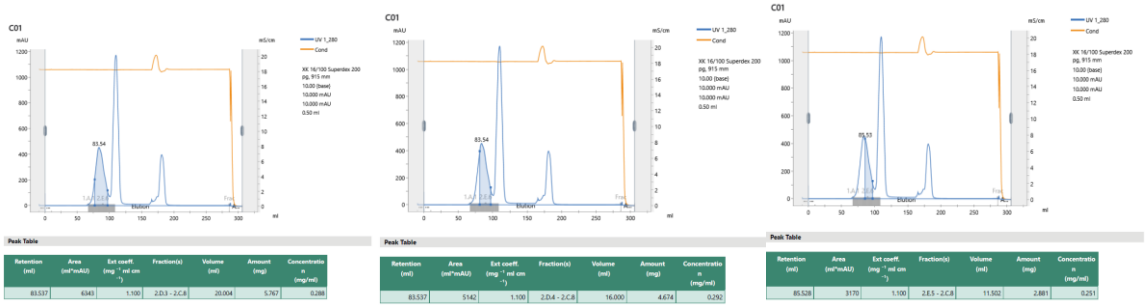


Figure 9. Pooled fractions for C01-A, C01-B and C01-C respectively.

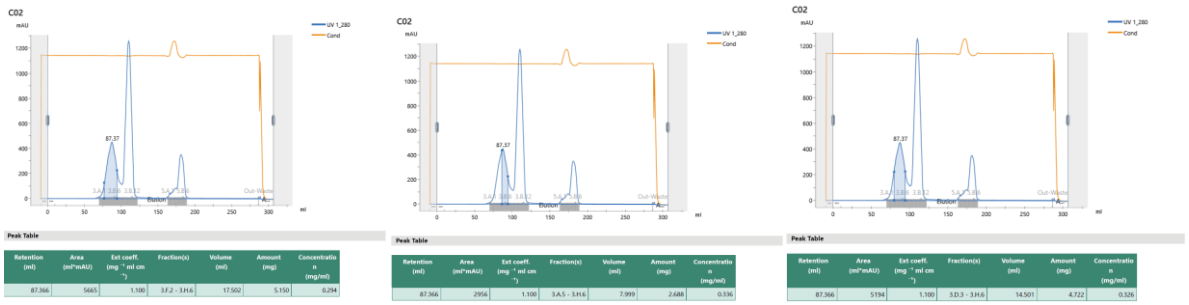


Figure 10. Pooled fractions for C02-A, C02-B and C02-C respectively.

Immunoassay with the Reagents

As mentioned, five combinations of antibody pairs were created and analysed in the project. Fraction pools C01-B and C02-B were chosen as the conjugates due to prior knowledge of functioning conjugate size in other FDAB projects. All four antibodies were used as capture antibodies in one or two assay runs. Duplicates of every sample were run and the mean value of these was calculated for every sample.

The calibration curves and controls were processed in the Excel-based tool which returned a suitable curve fit followed by the concentration levels of the controls based on the calibration curve (**Table 7**).

Table 7. RLU response of the five runs

	Run	**Run 1**	**Run 2**	**Run 3**	**Run 4**	**Run 5**
	Capture Ab	NFL02	NFL01	NFL03	NFL04	NFL02
	Detection Ab	NFL01	NFL02	NFL02	NFL01	NFL02
CAL ID	**Lumipulse Conc (pg/mL)**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**
S0	4	**12 524**	**28 700**	**11 679**	**38 500**	**19 028**
S1	43	48 362	31 787	67 711	55 260	31 316
S2	399	310 310	39 336	500 575	199 402	121 433
S3	630	470 967	42 045	753 837	274 938	156 462
S4	2 195	1 558 770	76 440	2 333 360	816 593	503 858
S5	4 448	2 951 400	110 655	4 723 765	1 647 820	1 075 818
S6	8 935	5 306 050	201 982	8 049 835	3 336 985	2 020 645
S7	25 206	15 415 500	563 976	21 494 400	9 941 475	6 196 535
S8	39 043	**22 068 400**	**817 763**	**31 938 600**	**15 661 200**	**11 098 250**
S1/S0		3.9	1.1	5.8	1.4	1.6

To investigate the sensitivity, the RLU values for S1/S0 were calculated which provides a signal-to-noise value. By inspecting the S1/S0 values, one cannot determine if the tests are successful, however it is useful for run-to-run comparison.

Controls

Duplicates of CTRL 1-13 were run for the five combinations with RLU responses calculated to dose values (**Table 8**). The recovery percentage versus the predicate device was calculated and stated in Table 8. Run 1 showed comparable results to run 3. Run 2 showed high recovery

rates for most controls except the patient samples. Run 4 and 5 showed the lowest recovery rates with the latter being the absolute lowest. CTRL 1, 2, 8 and 9 (bovine NfL and calibration matrix prepared at FDAB) showed medium to high recovery in all runs where the patient pools and samples generated low recoveries in most runs except for runs 1 and 3.

Table 8. Mean concentration of the controls and the Lumipulse concentration recovery

			Run	Run 1		Run 2		Run 3		Run 4		Run 5	
			Capture Ab	NFL02		NFL01		NFL03		NFL04		NFL02	
			Detection Ab	NFL01		NFL02		NFL02		NFL01		NFL02	
CTRL ID	**Target**	**Antigen**	**Matrix**	**Lumipulse Conc (pg/mL)**	**Dose (pg/mL)**	**%dose recovery vs LP**	**Dose (pg/mL)**	**%dose recovery vs LP**	**Dose (pg/mL)**	**%dose recovery vs LP**	**Dose (pg/mL)**	**%dose recovery vs LP**	**Dose (pg/mL)**	**%dose recovery vs LP**
CTRL3	787	Lumipulse Antigen	Lumipulse matrix	762	1025	130	746	95	970	123	392	50	22	3
CTRL7	4 056	Lumipulse Antigen	Lumipulse matrix	4 060	5115	126	3508	86	5172	128	2035	50	103	3
CTRL10	21 010	Lumipulse Antigen	Lumipulse matrix	20 824	23301	111	19035	91	26011	124	11579	55	468	2
CTRL1	200	Bovine NfL	Calibration matrix	158	96	48	322	161	102	51	170	85	163	81
CTRL2	400	Bovine NfL	Calibration matrix	313	306	77	313	78	284	71	344	86	322	80
CTRL8	6 050	Bovine NfL	Calibration matrix	4 416	4314	71	6983	115	4197	69	4447	74	4841	80
CTRL9	20 000	Bovine NfL	CSF	16 818	16680	83	15690	78	17202	86	17152	86	18667	93
CTRL11	2 100	N/A	CSF bulk	1 661	1143	54	0	0	2011	96	192	9	87	4
CTRL4	1 287	Patient pool	CSF	997	955	74	0	0	1769	137	206	16	54	4
CTRL5	1 710	Patient pool	CSF	1 260	621	36	168	10	1216	71	154	9	35	2
CTRL6	2 070	Patient pool	CSF	1 395	846	41	0	0	1593	77	188	9	36	2
CTRL12*	4718*	Patient sample	CSF	N/A	1853	N/A	0	N/A	N/A	N/A	355	N/A	N/A	N/A
CTRL13*	> 5000*	Patient sample	CSF	N/A	3257	N/A	0	N/A	N/A	N/A	651	N/A	N/A	N/A

* Values from Uman Diagnostic's NfL Assay.

Discussion

Calibration Curve

The first calibration curve presented, i.e., the Lumipulse calibration curve, showed similar values as the target values set in this study, with some variation. This shows that the preparation of the calibrators was successful. For other CSF NfL immunological tests (including Uman Diagnostics etc) the highest calibration level is around 5 000 pg/mL which is significantly lower than the highest calibration level in this study, S8 (50 000 pg/mL). The choice of calibrator values was highly dependent on the calibrator levels of the predicate device since it was used as comparison. However, it was important to include lower calibration levels between 0 and 5 000 pg/mL for clinical relevance of the test. The cut-off values for healthy patients are around 500 pg/mL depending on age. The Lumipulse device is a CLIA, while Uman Diagnostics is an ELISA which also explains the different calibration curve ranges between the two.

The calibration levels consist of Bovine NfL diluted with a calibration matrix which is an important note when discussing the results of the five CLIA runs. Evidentially, the results of the four controls of the same composition (CTRL1, 2, 8, and 9) showed high recovery where other controls showed very low recovery percentage.

PMP Coating

PMPs are chosen as capture Ab conjugate due to the design of both the automated device and the predicate device. The interaction between the PMPs and the magnets in the devices are essential during the washes which makes the PMPs one of few useful capture conjugates. In other devices and assays, other particles such as gold particles can be used, as well as Abs coated with streptavidin. By the experiments performed in this study, the capture Ab functionalities and performances are hard to determine. However, by studying the sedimentation of the PMP Ab complexes during the coating procedure, one can get a hint of how stable they are. In this study, all capturing Abs showed low to no sedimentation which may imply that the buffers and agents used in the PMP coating procedure were suitable.

Conjugation

The results from the monomer ratio analysis by the UHPLC-SEC show that the desalted thiolated antibodies (DS SH-Ab) exhibit higher stability than the not desalted ones, for all antibodies in the AHTL titration experiment (NFL01, NFL02, and NFL03). This can be explained by the fact that the desalted compounds are detained in an optimised buffer and that potential rests of AHTL have been more extensively separated from the antibody-thiol compounds. Thiolated NFL03 appears to be the complex least prone to aggregation. NFL03 could therefore be a promising contender to being a detection Ab after ALP conjugation, which could be further explored in future experiments. NFL02 showed to be the least stable antibody of the three displaying significant decreases in peak area during the varying conditions. In the assay runs, NFL02 shows both promising and unfavourable results as a detection Ab which implies that other properties than solely Ab-thiol complex stability is at cause. NFL02 also displays curious results where the stability increases from day one to day two, at both room temperature and at 10°C. This would need further investigation. Altogether, the stability is higher for all antibodies when thiolated with lower AHTL concentration and thereby acquiring a lower thiolation degree. However, higher thiolation degrees provides the antibody with more sites to bind to the ALP molecule which has proven to yield better performing conjugates and amplified responses in CLIAs according to prior experiments at FDAB.

The size of the two conjugates, C01 and C02, can be discussed. The chosen pools of fractions were C01-B and C02-B. By choosing the medium sized conjugates, one makes sure to not lose to much antibody-ALP complex by avoiding the smallest conjugates. The large conjugates have higher tendencies to aggregate and were therefore voted out as well. With this mentioned, smaller or larger conjugate complexes could prove to be more successful in the assays than the medium ones. Evaluating the effect of the conjugate size in this experiment would require extensive testing of the different conjugates in the assay runs but is an interesting note for future experiments.

Comparison of Assay Background

By inspecting **Table 7**, one can see multiple differences in the RLU responses for the calibration levels S0-S8 generated by the five runs. The first value, S0, showed large run- to-run variation and can in this study be a measurement of background of the immunological test. Background can, as mentioned, be a result of multiple parameters and in this study, the values are mainly used for comparison between the different runs. The run- to- run background variation between run 1 and run 2 showed that even though the same antibodies were used, making the switch of detection and capture antibody can affect the results greatly. NFL02 as capture antibody and NFL01 as detection antibody showed much lower background signal than if they were reversed. However, it does not seem like NFL02 is a bad detection antibody since it provides low background in Run 3 as well as high RLU responses for the other calibration levels. Same goes for NFL01. In Run 1, NFL01 provides the second lowest background signal as a detecting Ab while providing the highest background signal in run 4, proving that the pairing of the antibodies has a big influence on the test results. The capture antibody may generate steric hindrance when bound to the antigen which can prevent binding of the detection Ab and thereby low detection responses.

In addition to inspecting the background of the runs, S1/S0 was applied. By dividing calibration level S1 with S0, one can get an implication of how specific the antibody-antigen bindings are. Again, these values are for comparison and cannot be used to determine if one test is successful or not. Two immunoassays showed higher S1/S0 values than the rest; run 1 and run 3. Run 2 showed the lowest S1/S0 value, yet again implying that a switch of the detecting and capturing antibody can result in highly varying results. Run 4 and 5 both showed high background and low S1/S0 which can imply that these two antibody pairs need further optimisation or simply do not interact well with each other or the antigen.

Seeing how the background signals and S1/S0 values of the different pairs behave one can conclude that the pairing of these antibodies is of great significance. It is also clear that the signalling differs depending on which antibody is detecting and which is capturing.

Comparison of Assay Recovery

Recovery to the Lumipulse doses is another one of this study's measurements for investigating the immunoassays' performances. Inspecting **Table 8**, one can find that the recoveries of the controls differ from assay to assay, but also control to control in each assay.

Comparing runs 1 and 2, differences can be seen in dose recovery. Again, switching the two antibodies NFL01 and NFL02 in run 2 provides the assay with much lower doses and control recoveries than in run 1, except CTRL 1, 2, and 8, the latter proving to be the inferior immunoassay of the two. Run 2 shows uneven results which may be a consequence of the low responses and shape of the calibration curve.

Using the same Ab as capturing and detecting Ab is something that theoretically does not work in immunoassays like these due to them binding to the same epitope of the antigen. However, other single antibody assay formats can work successfully [31]. Run 5 was performed as an experimental immunoassay. Looking at the recoveries of run 5, it is interesting that the assay showed RLU responses for the calibration curve but showed very low signalling for all controls except CTRL 1, 2, 8 and 9. All runs show good recoveries for these controls. The pattern of recovery deviation in all runs can be connected to the differences in antigen and matrix in the controls. These controls are manufactured the same way as the calibration curve which could explain some part of the high recoveries. Another thought could be that the bovine NfL antigen is produced and purified for these kinds of applications and thereby easier to locate and bind to than the human NfL in the patient samples. There could be post-translational differences between the bovine NfL antigen and the patient NfL, like glycosylation and phosphorylation as well as structure differences. The artificially made matrix is another potential parameter that might make the non-human controls more recognisable due to lower concentrations of other proteins in the solution. However, due to recent studies [4], [5], the dimerization tendency of NfL as well as their larger segments in bovine NfL than in human NfL, is the most promising reason for Run 5 to being able to show existing NfL concentration as an assay with two antibodies locating the same epitope.

This experiment shows that it is important to include controls of different origins when developing an immunological test. The patient samples show low to non-existing doses in runs 2, 4, and 5 which would not be acceptable when further developing the tests. This, together with the discussion about assay background, emphasises the importance of calibrators and controls of different origin in immunoassay development.

The pattern of recovery deviation run-to-run is, in contrast to what is discussed in the previous paragraph, not as obvious. Controls 3, 7, and 10, i.e., bovine NfL in Lumipulse Matrix, show high values in runs 1-3. Run 1 and 3 show the highest dose values with recoveries ranging between 112-135%. What does it say about these two assays, showing higher signal than Lumipulse? Are they more sensitive to the NfL antigen in the predicate matrix than their predicate device? For run 3, one could argue that the assay seems to show higher values than the predicate device and therefore is more sensitive than Lumipulse. Although, the overall high recovery rates can be a result of interference and be false positives. Run 1, on the other hand, displays slightly lower doses for the following controls which is interesting. Meanwhile, runs 4 and 5 display shallow recoveries for these three controls meaning that they are not functional for this kind of antigen.

Could the immunoassay results differ due to the different subtypes of antibodies and how they are paired or what function (detecting or capturing) they have? To evaluate if the pairing of different subtypes had an impact on the results, more combinations must be run. The five pairs are combinations of IgG1 with IgG2a or IgG2b, except for run 5 where to the same IgG2 type is combined (NFL02-NFL02), which makes it hard to say if IgG1 paired with IgG1or IgG2 paired with IgG2 would be better matches. Additionally, runs 1 and 3 show that subtype IgG1

functions both as detection Ab (NFL01 in run 1) and capture Ab (NFL03 in run 3) and that subtype IgG2b functions both as detection and capture Ab. Therefore, one cannot argue that it is the antibodies subtypes that determine the assay results, rather that the antibodies possess other properties which makes them successful for the cause as well as the pairing of them as previously discussed.

The antibodies used in this study have been evaluated in previous analytical processes which have proven their affinity for the NfL antigen. Yet, this study shows that regardless of how well your antibody binds to the protein, the suitable antibody companion must be applied for the immunoassay to be effective and show diagnostic potential.

Assay Setup

The immunological test developed in this study is a CLIA for NfL in CSF which performance is compared to a market-established CSF NfL CLIA instrument, Lumipulse. The choice of predicate devise is based on various reasons. While alternative CLIA assays are available, such as Siemens Atellica [32], Quanterix's Simoa [33] , and Roche's Elecsys [34], it is important to note that the latter two lack a validated NfL assay. Other NfL assays are at hand, prominently ELISA assays provided from manufacturers including Uman Diagnostics and Bio-Techne. While reports show that correlation between CLIA and ELISA results are agreeable [35] and others not [36], the best choice of predicate device was decided to be a CLIA Instrument with an established NfL CSF Assay and based on its availability, Lumipulse was a suitable choice.

The assay format was chosen to be a two-step assay in which the sample is first in contact with the capturing Abs. The choice of format was mostly dependent on the predicate device for complete comparison, but also due to its advantages. A two-step assay reduces interference compared to a one-step assay since the remaining components in the sample are washed away after the first binding between the antigen and the capture antibodies. One can optimise the time lasting of every step which may bring better results. However, a one-step immunoassay may contribute to a more sensitive response due to the antigen being longer exposed to two types of antibodies and the risk of washing away antigen reduces.

This study’s immunoassay specifically targets NfL biomarkers in CSF and not in blood. CSF is chosen due to its higher concentrations of neuro biomarkers, including NfL. Additionally, CSF assays are less vulnerable to assay interference due to lower protein and cellular component concentration in CSF samples compared to blood samples. However, the invasive nature of CSF sampling may result in limited sample availability which consequently may decelerate the development of tests relying on CSF samples. Therefore, it is important to consider the clinical objective of the test. For this study, CSF in a two-step assay was used due to the higher concentration of NfL and lesser interference which was preferable in a time-limited experiment. However, future optimization of test parameters such as blocking agents, assay format and more could permit the development of an immunological test for NfL in blood in the automated device.

There is potential for optimisation of this study's immunological test. The buffers used can be altered, both for the reagents, calibration curves and the assay run. Changing the pH value, salt concentrations, the amount, and kinds of detergents among multiple other factors could generate significant result improvements. The reagents can be optimised in various ways, including changing the thiolation degree of the detection Ab or the particle size of the PMPs. Other assay formats, such as one step or delayed one step, can be tested as well as testing of samples from larger and other patient groups. Further in the future comes the need of stability and performance testing and verification studies.

Conclusion

In conclusion, this study successfully achieved its objectives, which were centred around the development and evaluation of a CLIA towards the biomarker NfL in CSF. Several key aspects of the immunoassay were explored, including the calibration curve, PMP coating, thiolation, and ALP conjugation. Antibody-based reagents were successfully created with bioconjugation techniques. The discussion of assay background and assay recovery emphasised the influence of antibody choices and antibody paring although not establishing any choices as right or wrong. Two immunoassay runs proved to be more successful (runs 1 and 3) which could be further explored and optimised in future experiments.

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Appendix I

Table AI. Target standard values and predicate device standard values including standard deviation, %CV, and %Recovery between predicate and target values.

Sample	Target conc	Conc	Mean conc	STDV.S	CV	Recovery (%)
S0	0	4 4	4	0	0.00%	
S1	50	42 44	43	1.414214	3.29%	86%
S2	250	395 404	399	6.363961	1.59%	160%
S3	500	621 639	630	12.72792	2.02%	126%
S4	2 500	2 220 2 170	2195	35.35534	1.61%	88%
S5	5 000	4 403 4 493	4448	63.63961	1.43%	89%
S6	10 000	8 983 8 888	8935	67.17514	0.75%	89%
S7	30 000	25 084 25 329	25206	173.2412	0.69%	84%
S8	50 000	39 008 39 079	39043	50.20458	0.13%	78%

Appendix II

Degree of thiolation NFL01

Table AIIa. Absorbance values and calculated thiolation degree from AHTL Titration for Ab NFL01

	NFL01			
[AHTL] mM	Abs	Mean Abs	%CV	SH:Ab Ratio
5	0.054 0.055 0.054	0.054	1.1%	1.44
10	0.087 0.090 0.097	0.091	5.6%	2.42
15	0.171 0.174 0.173	0.173	0.9%	4.58

Degree of thiolation NFL02

Table AIIb. Absorbance values and calculated thiolation degree from AHTL Titration for Ab NFL02

	NFL02			
[AHTL] mM	Abs	Mean Abs	CV	SH:Ab Ratio
5	0.105 0.111 0.11	0.109	3.0%	2.88
10	0.149 0.148 0.149	0.149	0.4%	3.94
15	0.382 0.398 0.397	0.392	2.3%	10.40

Degree of thiolation NFL03

Table AIIc. Absorbance values and calculated thiolation degree from AHTL Titration for Ab NFL03

	NFL03			
[AHTL] mM	Abs	Mean Abs	%CV	SH:Ab Ratio
5	0.088 0.093 0.093	0.091	3.2%	2.42
10	0.111 0.113 0.115	0.113	1.8%	2.99
15	0.170 0.174 0.175	0.173	1.5%	4.58

Appendix III

UHPL-SEC Results from AHTL Titration of NFL01, NFL02, and NFL03

Table AIIIa. UHPLC-SEC results for NFL01

Injection Name	AHTL Concentration	Desalted/ Not desalted	Storage Temperature C	Storage Time	Ret.Time min UV_VIS_3 monomer	Area mAU*min UV_VIS_3 Monomer	Height mAU UV_VIS_3 Monomer	Rel.Area % UV_VIS_3 Monomer	Rel.Height % UV_VIS_3 Monomer	Column Injection #
NFL01	ELN388.2		N/A	D0	4,208	40,375	283,936	99,36	99,61	230
NFL01 in wash buffer	N/A	DS	10C	D1	4,217	22,996	161,447	99,34	99,59	253
NFL01 in wash buffer	N/A	DS	10C	D2	4,217	23,842	167,415	99,34	99,59	279
NFL01 in wash buffer	N/A	DS	N/A	D0	4,217	22,871	161,234	99,36	99,61	231
NFL01 in wash buffer	N/A	DS	RT	D1	4,208	22,852	160,485	99,23	99,55	254
NFL01 in wash buffer	N/A	DS	RT	D2	4,208	22,989	161,104	99,31	99,57	280
SH-NFL01	5 mM		10C	2H	4,208	18,704	124,242	98,17	99,01	238
SH-NFL01	5 mM		10C	D1	4,208	18,337	113,241	95,90	97,49	255
SH-NFL01	5 mM		10C	D2	4,208	18,076	105,894	94,00	96,26	281
SH-NFL01	5 mM		N/A	D0	4,208	18,806	127,177	98,45	99,19	232
SH-NFL01	5 mM	DS	10C	2H	4,208	10,827	74,798	97,69	98,48	241
SH-NFL01	5 mM	DS	10C	D1	4,217	10,891	74,992	96,76	97,72	258
SH-NFL01	5 mM	DS	10C	D2	4,217	11,154	76,844	95,92	96,98	284
SH-NFL01	5 mM	DS	N/A	D0	4,217	10,799	73,466	97,38	98,44	235
SH-NFL01	5 mM	DS	RT	3H	4,208	10,876	74,653	97,24	98,20	247
SH-NFL01	5 mM	DS	RT	D1	4,225	2,740	18,829	92,21	95,79	264
SH-NFL01	5 mM	DS	RT	D2	4,217	11,673	80,240	95,23	96,37	290
SH-NFL01	5 mM		RT	3H	4,208	18,650	124,770	98,12	98,97	244
SH-NFL01	5 mM		RT	D1	4,208	18,332	109,622	95,16	96,78	261
SH-NFL01	5 mM		RT	D2	4,208	18,252	107,149	94,52	96,58	287
SH-NFL01	10 mM		10C	2H	4,208	18,160	111,569	94,31	97,08	239
SH-NFL01	10 mM		10C	D1	4,217	17,025	96,457	87,45	93,41	256
SH-NFL01	10 mM		10C	D2	4,217	16,238	90,052	82,86	88,02	282
SH-NFL01	10 mM		N/A	D0	4,225	5,592	35,580	93,71	96,97	233
SH-NFL01	10 mM	DS	10C	2H	4,217	8,038	52,036	87,68	90,71	242
SH-NFL01	10 mM	DS	10C	D1	4,217	7,954	51,385	86,65	89,72	259
SH-NFL01	10 mM	DS	10C	D2	4,217	7,990	51,482	85,65	88,78	285
SH-NFL01	10 mM	DS	N/A	D0	4,217	8,003	51,252	87,35	90,70	236
SH-NFL01	10 mM	DS	RT	3H	4,217	8,035	51,614	87,20	90,23	248
SH-NFL01	10 mM	DS	RT	D1	4,217	7,893	50,620	85,73	88,85	265
SH-NFL01	10 mM	DS	RT	D2	4,217	7,834	50,324	84,68	87,89	291
SH-NFL01	10 mM		RT	3H	4,208	17,979	108,736	93,41	96,48	245
SH-NFL01	10 mM		RT	D1	4,208	16,355	92,950	83,40	91,34	262
SH-NFL01	10 mM		RT	D2	4,208	17,189	95,849	83,04	91,12	288

SH-NFL01	15 mM		10C	2H	4,217	15,790	94,355	87,21	91,81	240
SH-NFL01	15 mM		10C	D1	4,217	14,872	84,974	81,08	87,46	257
SH-NFL01	15 mM		10C	D2	4,217	14,596	81,141	78,44	85,18	283
SH-NFL01	15 mM		N/A	D0	4,217	15,891	94,445	87,71	92,13	234
SH-NFL01	15 mM	DS	10C	2H	4,217	7,817	45,716	84,66	90,10	243
SH-NFL01	15 mM	DS	10C	D1	4,217	7,942	46,423	84,68	89,83	260
SH-NFL01	15 mM	DS	10C	D2	4,217	8,200	48,020	84,06	89,13	286
SH-NFL01	15 mM	DS	N/A	D0	4,217	7,799	45,673	84,26	90,09	237
SH-NFL01	15 mM	DS	RT	3H	4,217	0,051	0,310	17,65	34,46	249
SH-NFL01	15 mM	DS	RT	D1	4,217	7,825	45,211	84,10	89,31	266
SH-NFL01	15 mM	DS	RT	D2	4,217	7,890	45,800	84,59	89,23	292
SH-NFL01	15 mM		RT	3H	4,217	15,540	92,054	85,71	90,79	246
SH-NFL01	15 mM		RT	D1	4,208	14,545	82,426	79,24	85,98	263
SH-NFL01	15 mM		RT	D2	4,208	14,967	83,178	80,24	86,47	289

Table AIIIb. UHPLC-SEC results for thiolated NFL02 (AHTL Titration)

Injection Name	AHTL Concentration	Desalted/ Not desalted	Storage Temperature C	Storage Time	Ret.Time min UV_VIS_3 monomer	Area mAU*min UV_VIS_3 Monomer	Height mAU UV_VIS_3 Monomer	Rel.Area % UV_VIS_3 Monomer	Rel.Height % UV_VIS_3 Monomer
NFL02			N/A	D0	4,083	20,910	140,194	98,29	99,06
NFL02 in wash buffer	N/A	DS	10C	D1	4,083	13,704	91,710	97,89	98,85
NFL02 in wash buffer	N/A	DS	10C	D2	4,083	13,930	92,831	97,83	98,81
NFL02 in wash buffer	N/A	DS	N/A	D0	4,083	13,502	90,262	98,12	98,96
NFL02 in wash buffer	N/A	DS	RT	D1	4,083	13,557	90,051	98,01	98,92
SH-NFL02	5 mM		10C	D1	4,100	10,651	51,443	90,54	93,84
SH-NFL02	5 mM		10C	D2	4,092	10,316	48,077	85,77	87,16
SH-NFL02	5 mM		N/A	D0	4,083	10,673	60,002	92,52	95,81
SH-NFL02	5 mM	DS	10C	D1	4,092	7,225	42,988	93,27	96,10
SH-NFL02	5 mM	DS	10C	D2	4,092	7,264	43,353	92,70	95,65
SH-NFL02	5 mM	DS	N/A	D0	4,083	7,255	42,584	93,61	96,55
SH-NFL02	5 mM	DS	RT	D1	4,092	7,172	42,398	93,16	95,77
SH-NFL02	5 mM	DS	RT	D2	4,092	7,235	43,168	92,05	94,95
SH-NFL02	5 mM		RT	D1	4,092	10,586	52,910	90,50	94,19
SH-NFL02	5 mM		RT	D2	4,100	0,056	0,296	23,19	41,04
SH-NFL02	10 mM		10C	D1	4,125	9,165	38,135	78,27	79,99
SH-NFL02	10 mM		10C	D2	4,125	8,635	35,145	73,25	75,39
SH-NFL02	10 mM		N/A	D0	4,092	9,312	45,270	79,78	83,42
SH-NFL02	10 mM	DS	10C	D1	4,100	5,945	29,931	84,27	91,24
SH-NFL02	10 mM	DS	10C	D2	4,100	5,992	30,781	84,42	91,70
SH-NFL02	10 mM	DS	N/A	D0	4,092	5,868	29,055	82,76	89,89
SH-NFL02	10 mM	DS	RT	D1	4,100	5,913	29,698	83,95	91,06
SH-NFL02	10 mM	DS	RT	D2	4,100	5,970	30,478	84,29	90,81
SH-NFL02	10 mM		RT	D1	4,108	9,121	39,137	76,73	79,05
SH-NFL02	10 mM		RT	D2	4,092	8,601	37,472	72,33	74,98
SH-NFL02	15 mM		10C	D1	4,150	8,601	35,444	72,03	75,29
SH-NFL02	15 mM		10C	D2	4,142	8,415	33,644	68,87	72,29
SH-NFL02	15 mM		N/A	D0	4,108	8,321	37,534	71,35	75,70
SH-NFL02	15 mM	DS	10C	D1	4,108	5,794	26,003	73,83	76,43
SH-NFL02	15 mM	DS	10C	D2	4,117	5,939	26,991	75,30	77,85
SH-NFL02	15 mM	DS	N/A	D0	4,108	5,654	25,130	71,70	74,93
SH-NFL02	15 mM	DS	RT	D1	4,108	5,843	26,074	74,69	76,78
SH-NFL02	15 mM	DS	RT	D2	4,117	6,040	27,475	76,57	86,44
SH-NFL02	15 mM		RT	D1	4,133	8,406	34,865	70,16	73,82
SH-NFL02	15 mM		RT	D2	4,108	8,093	33,837	67,56	71,22

Table AIIIc. UHPLC-SEC results for thiolated NFL03 (AHTL Titration)

Injection Name	AHTL Concentration	Desalted/Not desalted	Storage Temperature C	Storage Time	Ret.Time min UV_VIS_3 monomer	Area mAU*min UV_VIS_3 Monomer	Height mAU UV_VIS_3 Monomer	Rel.Area % UV_VIS_3 Monomer	Rel.Height % UV_VIS_3 Monomer	Comments
NFL03			N/A	D0	4.242	25.904	188.31	97.46	97.72	
NFL03			N/A	D1	4.242	25.979	189.137	97.46	97.73	
NFL03			N/A	D2	4.242	26.162	190.546	97.45	97.74	
NFL03 in wash buffer	N/A	DS	10C	D1	4.242	14.612	105.834	97.66	97.99	
NFL03 in wash buffer	N/A	DS	10C	D2	4.242	14.671	106.534	97.63	98	
NFL03 in wash buffer	N/A	DS	N/A	D0	4.242	14.609	106.059	97.61	97.96	
NFL03 in wash buffer	N/A	DS	RT	D1	4.242	14.739	107.076	98.04	98.41	
NFL03 in wash buffer	N/A	DS	RT	D2	4.233	14.773	106.85	98.49	98.74	
SH-NFL03	5 mM		10C	D1	4.242	11.279	74.95	96.22	97.78	
SH-NFL03	5 mM		10C	D2	4.242	11.202	73.051	95.59	97.49	
SH-NFL03	5 mM		N/A	D0	4.233	0.016	0.124	49.53	65.89	Air injected. Rerun below
SH-NFL03	5 mM		N/A	D0	4.242	11.477	81.299	97.55	98.12	
SH-NFL03	5 mM	DS	10C	D1	4.242	5.162	36.816	96.63	97.2	
SH-NFL03	5 mM	DS	10C	D2	4.242	5.163	36.723	95	96.06	
SH-NFL03	5 mM	DS	N/A	D0	4.242	5.193	37.03	96.77	97.53	
SH-NFL03	5 mM	DS	RT	D1	4.242	5.184	36.914	96.24	97.25	
SH-NFL03	5 mM	DS	RT	D2	4.242	5.319	37.828	96.09	96.99	
SH-NFL03	5 mM		RT	D1	4.242	11.371	74.177	95.76	97.59	
SH-NFL03	5 mM		RT	D2	4.242	11.454	74.174	96.08	97.78	
SH-NFL03	10 mM		10C	D1	4.242	11.157	66.653	92.67	95.41	
SH-NFL03	10 mM		10C	D2	4.242	10.902	62.657	89.85	93.31	
SH-NFL03	10 mM		N/A	D0	4.242	11.412	73.666	95.1	97.13	
SH-NFL03	10 mM	DS	10C	D1	N/A	N/A	N/A	N/A	N/A	Aborted and rerun below
SH-NFL03	10 mM	DS	10C	D1	4.242	4.569	30.918	94.89	96.24	
SH-NFL03	10 mM	DS	10C	D2	4.242	4.552	30.904	94.16	95.58	
SH-NFL03	10 mM	DS	N/A	D0	4.242	4.614	31.59	96.02	97.14	
SH-NFL03	10 mM	DS	RT	D1	4.242	4.643	31.434	95.19	96.48	
SH-NFL03	10 mM	DS	RT	D2	4.242	4.625	31.291	94.43	95.85	
SH-NFL03	10 mM		RT	D1	4.242	10.963	62.686	89.1	92.77	
SH-NFL03	10 mM		RT	D2	4.242	11.101	63.443	90.87	93.88	
SH-NFL03	15 mM		10C	D1	4.25	10.127	57.546	85.39	90.56	
SH-NFL03	15 mM		10C	D2	4.25	10.231	57.82	85.31	90.54	
SH-NFL03	15 mM		N/A	D0	4.242	10.71	64.057	92.27	95.41	
SH-NFL03	15 mM	DS	10C	D1	4.242	4.418	27.549	93.97	96.21	
SH-NFL03	15 mM	DS	10C	D2	4.242	4.423	28.345	95.04	96.53	
SH-NFL03	15 mM	DS	N/A	D0	4.242	4.475	28.483	95.33	97.11	
SH-NFL03	15 mM	DS	RT	D1	4.242	4.509	28.247	94.21	96.32	
SH-NFL03	15 mM	DS	RT	D2	4.242	3.547	22.76	94.34	96.4	
SH-NFL03	15 mM		RT	D1	4.25	10.481	60.159	86.4	91.34	
SH-NFL03	15 mM		RT	D2	4.242	10.471	58.223	86.66	91.27	